Molecular Dissection of Lipid Biosynthetic Machinery in Green Algae, *Scenedesmus* species

SYNOPSIS

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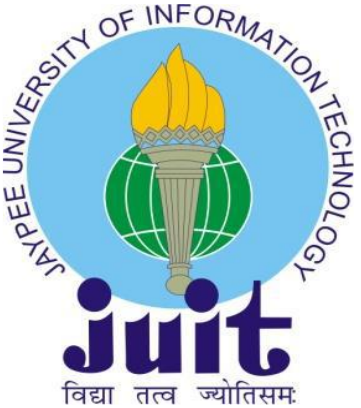

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MOLECULAR DISSECTION OF LIPID BIOSYNTHETIC MACHINERY IN GREEN ALGAE, *SCENEDESMUS* SPECIES

TAMANNA SHARMA

Introduction

With the depletion of petroleum-based diesel fuel, finding an alternative fuel resource to fulfill the energy demands of world is an important challenge. Biodiesel has gained preference over conventional fossil-based diesel as it provides advantages such as replacement of declining fossil fuel resources, reduced air pollution and greenhouse gas emissions [1]. Biodiesel is produced from edible or non-edible plant oils as well as animal fats. Production of biodiesel from edible plants competes with the food market, therefore makes it expensive surpassing the other benefits of biodiesel [2]. For sustainable biodiesel production, research focus should be towards production of biodiesel from non-food crops, organic fraction of urban waste and algae-based feedstock [3]. Microalgal feedstocks have recently emerged as the most promising alternative to produce biodiesel due to their higher lipid content and rapid biomass production [4]. Microalgae are aquatic photosynthetic organisms that possess the ability to convert sunlight, water and CO_2 into algal biomass [5].

Lipid metabolism in algae is analogous to higher plants including de novo fatty acid biosynthesis and triacylglycerol biosynthesis. Fatty acid (FA) biosynthesis occurs in plastid and triacylglycerol (TAG) biosynthesis in endoplasmic reticulum. Synthesized TAG molecules are accumulated in densely packed lipid bodies located in the cytoplasm of the microalgal cell [6].

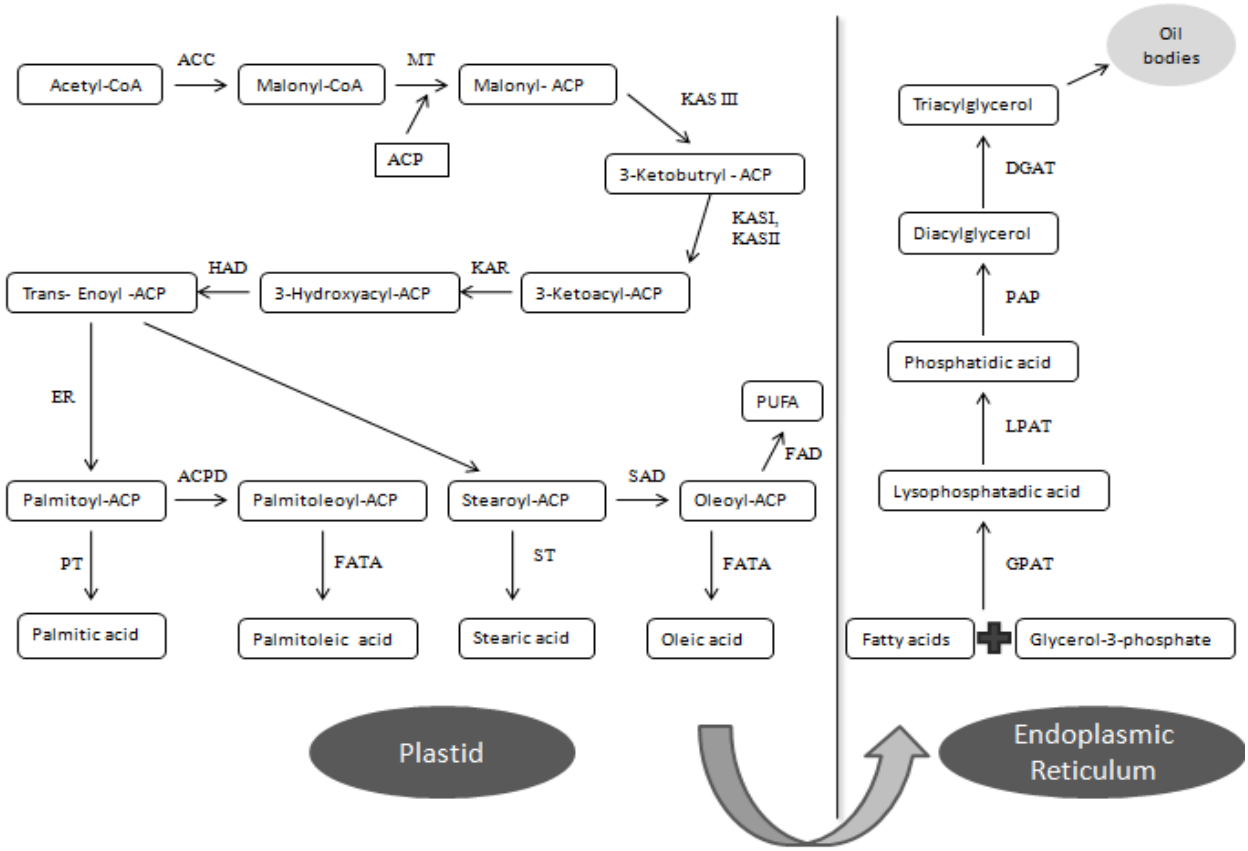

Fig. 1 The general scheme of microalgal fatty acid and triacylglycerol biosynthetic pathway [7, 8]. Enzyme abbreviations: ACC- Acetyl-CoA Carboxylase;MT- MalonylTransferase; ACP-Acyl Carrier Protein; KASI- β-ketoacyl-ACP synthaseI; ; KASII- β-ketoacyl-ACP synthase II; KASIII- β-ketoacyl-ACP synthaseIII; KAR- β-ketoacyl-ACP reductase; HAD- β-hydroxyacyl ACP dehydratase; ER- enoyl-ACP reductase; PT- Palmitoyl Thioestrase; FATA- Fatty Acyl-ACP Thioestrase; ST- Stearoyl Thioesterase; SAD- Stearoyl-ACP Desaturase; ACPD- Palmitoyl Desaturase; FAD- omega fatty acid desaturase; GPAT, glycerol-3-phosphate acyltransferase; LPAAT- Lyso-phosphatidic acid acyltransferase; PAP- Phosphatidic acid; DGAT- Diacylglycerolacyl transferase.

Commercial production of microalgal biodiesel is hindered by limitations in the productivity of strains and harvesting processes. Ideal strain for overall success of biodiesel production from microalgae would be having the combination of traits of high lipid content and fast growth rate. Microalgae synthesize lipids in normal conditions but under environmental stress conditions (mainly nutrient deprivation), lipid content of many microalgae increases up to several folds. However, increase in lipid content under stress conditions is associated with reduction in biomass productivity of microalgal cells [9]. Lipid content of microalgae varies among different

species and strains. Different strains of the same species have shown differences in lipid content and fatty acid composition, thereby, having variation in storage lipid metabolism among microalgal species [10-13]. In order to practically employ microalgae for biodiesel production, enhancing oil content without compromising growth is the straight-forward approach. Molecular understanding of lipid biosynthetic pathways and the regulatory mechanisms are least studied in algal species belonging to same genus but differing in their lipid accumulation potential so as to undertake suitable genetic interventions.

Although, regulation of genes involved in the storage lipid accumulation has been studied for various stress conditions in microalgae like *Haematococcus pluvialis* [14] and *Chlorella pyrenoidosa* [15]. Transcriptomes of various microalgal species, *Chlamydomonas reinhardtii, Neochloris oleoabundans, Dunaliella tertiolecta, Chlorella protothecoides, Dunaliella parva* were sequenced and compared under contrasting conditions of nutrient replete and nutrient deplete conditions to reveal the pathways and genes associated with high lipid content [16-20]. Little information is available on the transcriptional regulation of storage lipid biosynthesis in microalgae. Availability of genomic resources has led to the identification of transcription factors in microalgal species *Chlamydomonas reinhardtii* and *Nannochloropsis oceanica* [21, 22].

Scenedesmus is a freshwater green microalgae belongs to Phylum Chlorophyta, Class Chlorophyceae and Family Scenedesmaceae, has gained attention for its use in biodiesel production due to its high biomass productivity with high lipid content and desired fatty acid profile among green algae [23-25]. Lipid content variation has been reported among different species of *Scenedesmus* with 16 to 40% in *Scenedesmus. dimoprhus* and 1.9 to 18% in *Scenedesmus. quadricauda* [15, 25]

Thus, to ascertain the role of FA and TAG biosynthetic pathway genes for storage lipid production, cloning and relative gene expression analysis of 14 genes of fatty acid biosynthesis and 4 genes of triacylglycerol biosynthesis pathway was performed in various growth stages and stress conditions of *Scenedesmus* strains varying in their lipid content to identify key genes responsible for high lipid accumulation. Further, to elucidate the role of other metabolic pathways and regulatory mechanisms for differential lipid accumulation between species of the same genera of microalgae, transcriptomes of the two species of *Scenedesmus* i.e. *S. dimoprhus* and *S. quadricauda* at lipid accumulating phase were sequenced using NextSeq 500 platform of Illumina Sequencing. Comparative analysis of the various pathways and genes involved in storage lipid accumulation and biosynthesis of precursors for storage lipid biosynthesis was performed. Photosynthesis, carbon metabolism, fatty acid and triacylgycerol metabolism and catabolism were the main pathways studied. Further, transcription factors were identified from the transcriptomes of both the species, of which transcript abundance of lipid-related transcription factors (identified from literature) was estimated, which provided correlation of transcription factors with the high lipid accumulation. The current study was, therefore, undertaken with the following objectives:

Objective 1

Cloning and expression analysis of genes involved in lipid biosynthesis vis-à-vis lipid content in *Scenedesmus* species

Objective 2

Deciphering molecular components channeling precursors to differential lipid accumulation and their regulation in *Scenedesmus* species.

Objective 1-Cloning and expression analysis of genes involved in lipid biosynthesis vis-à-vis lipid content in *Scenedesmus* species

Materials and methods

Culturing of Scenedemus species/strains

Cultures of *S. dimoprhus* and *S. quadricauda* were maintained in the glass house of the Jaypee University of Information Technology, Waknaghat, H.P., India, under optimal conditions of light and temperature and grown in BG11media. Axenic cultures of both the species were obtained by treating the cultures with antibiotics: penicillin, streptomycin and chloramphenicol following the protocol of Guillard [26]. Cultures were harvested after 10, 18 and 24 days of incubation. For inducing nitrogen stress, cultures were suspended in BG11 medium containing KNO_3 as nitrogen source with concentration 2.5mM and for high salinity condition, the medium was supplemented with 180 mM NaCl. These cultures were harvested after 24 days of incubation and stored at−80 °C until further use.

Estimation of lipid content and biomass in different strains of S. dimoprhus and S. quadricauda

Lipid extraction from microalgal strains was carried out as described by Bligh and Dyer [27]. Total lipid content was calculated as percentage of the total biomass (in % dry weight). Optical densities of the microalgal suspension cultures were measured as absorbance at 730 nm using a spectrophotometer (ELICO SL-159 UV–VIS) to quantify microalgal biomass.

Genomic DNA and RNA extraction

Genomic DNA was isolated from the cultures of *Scenedesmus* strains using CTAB extraction protocol [28]. Total RNA was isolated using RaFlex RNA isolation kit (GeNei™) following the manufacturer's instructions. The quality of DNA and RNA was checked in 0.8% and 1% (w/v)

ethidium bromide-stained agarose gel and through the absorbance spectrum at wavelengths 260 nm and 280 nm. cDNA was prepared from 5 µg of RNA (RNA was treated with 2 U of DNase I).

Cloning and sequencing of fatty acid (FA) and triacylglycerol (TAG) biosynthetic pathway genes in S. dimoprhus

The nucleotide and protein sequences of five genes stearoyl-ACP desaturase (*SAD*), omega fatty acid desaturase (*FAD*), palmitoyl desaturase (*ACPD*), fatty acyl-ACP thioesterase (*FATA*), and enoyl-ACP reductase (*ER*) of different microalgal and plant species were retrieved from NCBI (http://www.ncbi.nlm.nih.gov) and aligned using ClustalW to find conserved regions. Primers were designed from the conserved regions using Primer3 and were amplified on the genomic DNA and cDNA of *S. dimoprhus*. The PCR products were cloned in pGEM-T vector (Promega) and sequenced. BLAST (http://www.ncbi.nlm.nih.gov/Blast) was used to calculate the identities of sequence similarities. For expression analysis, primers were designed from *S. dimoprhus* gene sequences using Primer3.

Transcriptome mining of genes involved in FA and TAG biosynthesis

The transcriptome data generated for *S. dimoprhus* were mined for nine genes, acetyl-CoA carboxylase (*ACC*), acyl carrier protein (*ACP*), Malonyl Transferase (*MT*), β-ketoacyl-ACP synthase I (*KASI*), β-ketoacyl-ACP synthase II (*KASII*), β-ketoacyl-ACP synthase III (*KASIII*), β-ketoacyl-ACP reductase (*KAR*), β-hydroxyacyl ACP dehydratase (*HAD*), palmitoyl thioesterase (*PT*) from fatty acid biosynthesis and four genes glycerol-3-phosphate acyltransferase (*GPAT*), lyso-phosphatidic acid acyltransferase (*LPAAT*), phosphatidic acid phosphatase (*PAP*), diacylglycerolacyl transferase (*DGAT*) of triacylgycerol biosynthesis. Primers were designed for these genes and amplified on the cDNA of *S. dimoprhus*.

RT- qPCR analysis

The qPCR was performed using gene specific primers in triplicate on a qPCR system (CFX96, Bio-Rad Laboratories; Hercules CA) with the iScript RT PCR kit (Bio-Rad). The PCR protocol was as follows: denaturation for 5 min at 94 °C, followed by 35 cycles each of denaturation for 20 s at 94 °C, annealing for 30 s at 50–55 °C, followed by one elongation step for 20 s at 72 °C.

Statistical analysis

Principal component analysis (PCA)

PCA was performed to determine correlation between three strains of *Scenedesmus* sp. i.e. SD16, SD12, SQ19 and their assessments in growth stages for eighteen genes of the fatty acid and triacylglycerol biosynthetic pathway.

Heat map

The heat map was made for RT-qPCR data to determine relative expression of FA and TAG biosynthetic pathway genes across different growth stages and stress conditions among three contrasting strains of *Scenedesmus* using GenEx software (V 1.1).

Results and discussion

Differential conditions for lipid accumulation in strains of Scenedesmus spp.

High lipid content strain SD12 (26%) and low lipid content strain SD16 (5%) from *S. dimoprhus* and one strain SQ19 of *S. quadricauda* having lipid content of 14% were considered for expression analysis (Fig. 2). There are different developmental phases of microalgal growth in batch cultures. First is lag phase, when culture is transferred to the fresh media in which microalgae adapt to the new environmental conditions. Next is exponential phase in which cell density increases as a function of time. Then microalgal growth ceases and remains constant which is a stationary phase and then eventually declines [29]. In our study, it was observed that

optical density of cultures started to increase after the second day of incubation and reached maximum at day 18, then became constant (Fig. 2). Therefore, three growth stages i.e. exponential phase (EXP) at day 10, early stationary phase (ESP) at day 18 and late stationary phase (LSP) at day 24 along with two stress conditions i.e. nitrogen deficiency (ND) and high salinity (HS) at late stationary phase (LSP) were selected for expression analysis.

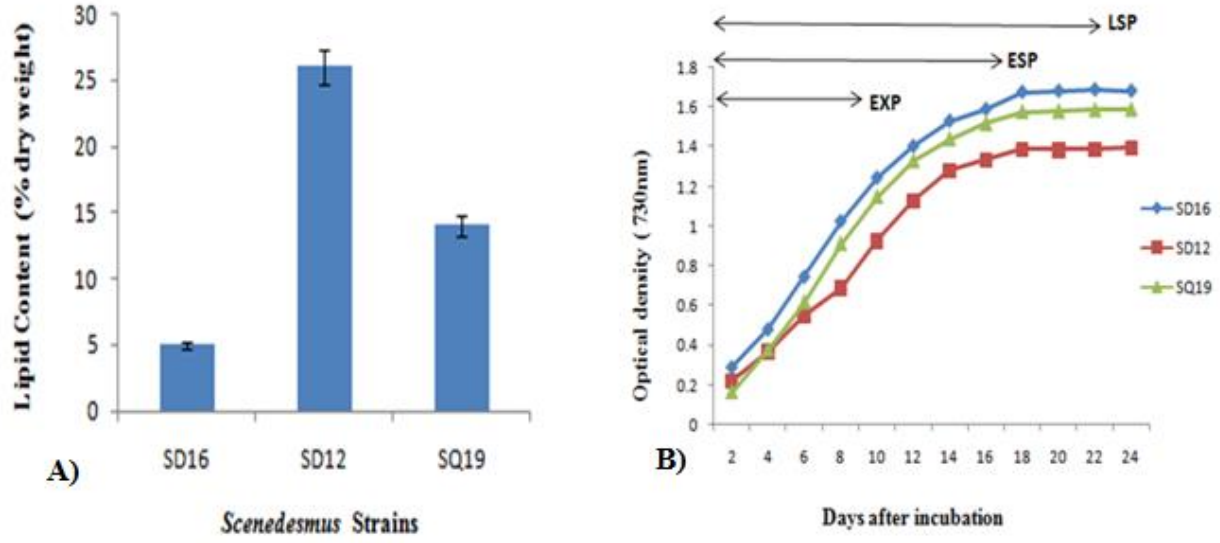

Fig. 2 A) Lipid content in three contrasting strains: *S. dimoprhus* (SD12, SD16) and *S. quadricauda* (SQ19) **B)** Biomass growth reflected through optical density of SD16, SD12 and SQ19 at 730 nm cultured in BG11 medium, Abbreviations: EXP — exponential phase, ESP — early stationary phase, LSP — late stationary phase.

Expression analysis of FA and TAG genes among three Scenedesmus strains

Comparative expression analysis among three lipid content contrasting strains of *Scenedesmus* revealed upregulation of majority of genes in the high lipid content strain SD12. It has been observed that genes involved in triacylglycerol biosynthesis (*LPAAT, PAP*, and *DGAT*) exhibited considerable fold elevation in high lipid content strain. This signifies that a greater level of control was exerted at triacylglycerol (TAG) biosynthesis than fatty acid (FA) biosynthesis

pathway in the regulation of storage lipid biosynthesis, which is supported by an earlier report in olive and oil palm [30].

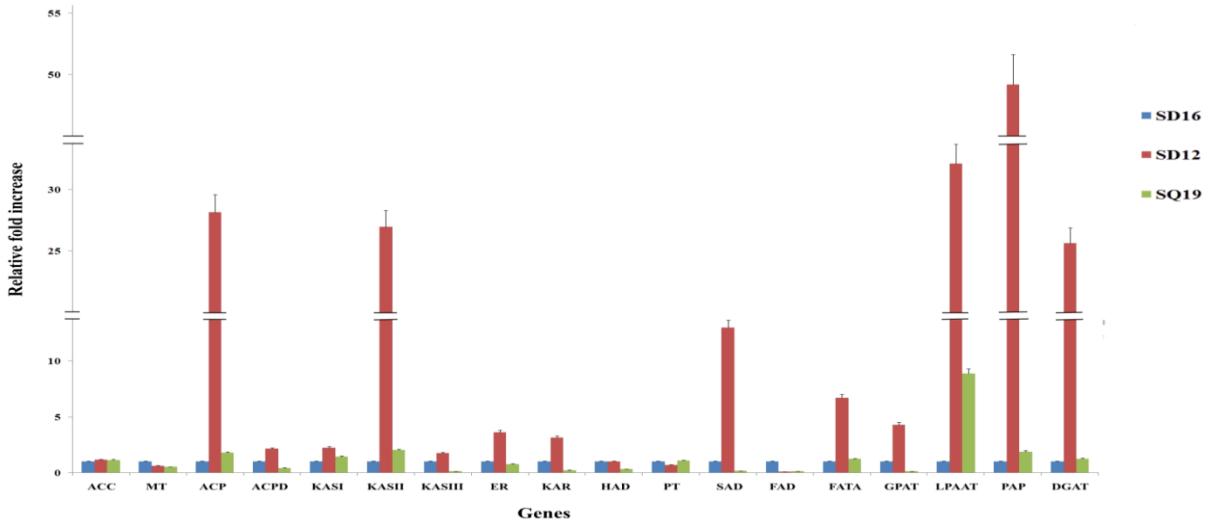

Fig. 3 Comparative account of expression of pathway genes across three lipid contrasting strains of *Scenedesmus*; SD12: *S. dimoprhus* 12; SQ19: *S. quadricauda* 19; SD16: *S. dimoprhus* 16 at late stationary phase (LSP). Enzyme abbreviations: ACC —acetyl-CoA carboxylase; MT—malonyl transferase; ACP — acyl carrier protein; KASI— β-ketoacyl-ACP synthase I; KASII —β-ketoacyl-ACP synthase II; KASIII — β-ketoacyl-ACP synthase III; KAR-β-ketoacyl-ACP reductase; HAD — β-hydroxyacyl ACP dehydratase; ER — enoyl-ACP reductase; PT — palmitoyl thioesterase; FATA — fatty acyl-ACP thioesterase; SAD — stearoyl-ACP desaturase; ACPD — palmitoyl desaturase; FAD — omega fatty acid desaturase; GPAT — glycerol-3-phosphate acyltransferase; LPAAT — lyso-phosphatidic acid acyltransferase; PAP — phosphatidic acid phosphatase; DGAT — diacylglycerolacyl transferase.

Gene expression variation with lipid accumulation

Genes *ACP, KASII, KASIII, ER, KAR, HAD, PT, SAD, FATA, LPAAT, PAP, DGAT* showed up regulation in lipid accumulating stage in all three strains (Fig. 4). Among 12 genes highly expressed in LSP, 4 genes are associated with de novo fatty acid biosynthesis (*ACP, KAR, HAD, ER*), two genes with condensation reactions in FA biosynthesis (*KASII, KASIII*), two are thioesterases (*PT, FATA*) that play role in terminating the fatty acid chain elongation, one desaturase gene *SAD* which incorporates double bond in saturated stearic acid to produce oleic acid and three genes associated with TAG biosynthesis (*LPAAT, PAP, DGAT*) [6, 31]. This

signifies that high transcripts of these genes might results in more accumulation of lipids in *Scenedesmus* species.

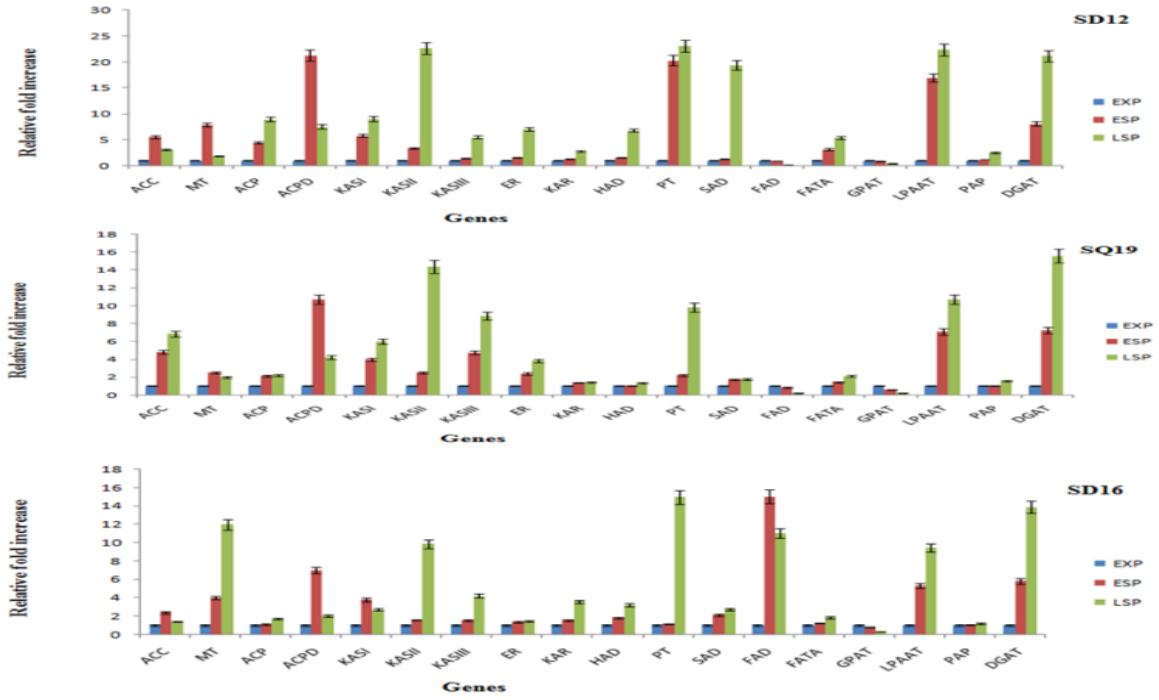

Fig. 4 Relative expression of FA and TAG biosynthetic in growth stages, ESP and LSP w.r.t EXP for three lipid contrasting strains of *Scenedesmus*; SD12: *S. dimoprhus* 12; SQ19: *S. quadricauda* 19; SD16: *S. dimoprhus* 16. Abbreviations: EXP—exponential phase, ESP—early stationary phase, LSP—late stationary phase, ACC — acetyl-CoA carboxylase; MT — malonyl transferase; ACP — acyl carrier protein; KASI — β-ketoacyl-ACP synthase I; KASII — β-ketoacyl-ACP synthase II; KASIII — β-ketoacyl-ACP synthase III; KAR — β-ketoacyl-ACP reductase; HAD — β-hydroxyacyl ACP dehydratase; ER — enoyl-ACP reductase; PT — palmitoyl thioesterase; FATA — fatty acyl-ACP thioesterase; SAD — stearoyl-ACP desaturase; ACPD — palmitoyl desaturase; FAD — omega fatty acid desaturase; GPAT — glycerol-3-phosphate acyltransferase; LPAAT — lyso-phosphatidic acid acyltransferase; PAP — phosphatidic acid phosphatase; DGAT —diacylglycerolacyl transferase.

Gene expression variation under stress conditions

Most of the genes showed increased expression in stress conditions with differences in the extent of elevation. Genes showing considerable increase in expression in cultures with one stress condition have lower abundance in the cultures exposed to other stress conditions. This signifies the role of multiple genes in regulating the mechanism of storage lipid accumulation as both the

stress conditions resulted in lipid content increase. Transcript abundance of *KASI* and *GPAT* decreased under both the stress conditions in all strains. It was reported earlier in *Chlamydomonas reinhardtii* that gene encoding *KASI* was down regulated under nitrogen deficiency and high salt stress [9].

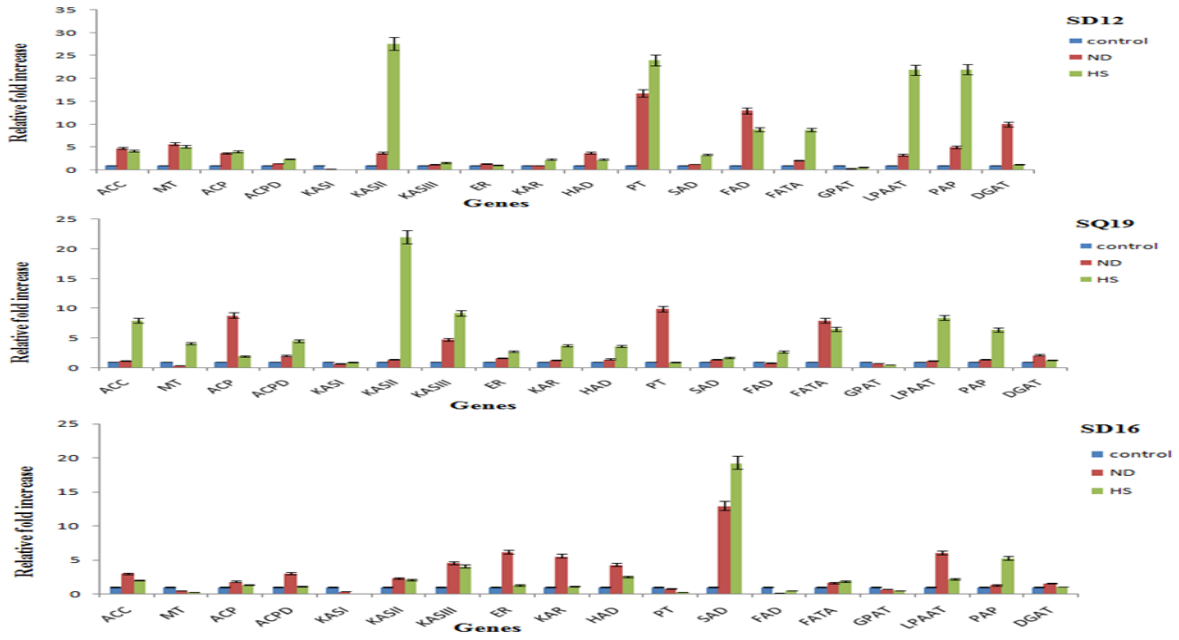

Fig. 5 Relative expression of FA and TAG biosynthetic genes under two stress conditions, ND and HS (at LSP) w.r.t LSP for three lipid contrasting strains of *Scenedesmus*; SD12: *S. dimoprhus* 12; SQ19: *S. quadricauda* 19; SD16: *S. dimoprhus* 16. Abbreviations: ND — nitrogen deficiency, HS — high salinity, ACC — acetyl-CoA carboxylase; MT — malonyl transferase; ACP — acyl carrier protein; KASI — β-ketoacyl-ACP synthase I; KASII — β-ketoacyl-ACP synthase II; KASIII — β-ketoacyl-ACP synthase III; KAR — β-ketoacyl-ACP reductase; HAD — β-hydroxyacyl ACP dehydratase; ER — enoyl-ACP reductase; PT — palmitoyl thioesterase; FATA — fatty acyl-ACP thioesterase; SAD — stearoyl-ACP desaturase; ACPD — palmitoyl desaturase; FAD — omega fatty acid desaturase; GPAT — glycerol-3-phosphate acyltransferase; LPAAT — lyso-phosphatidic acid acyltransferase; PAP — phosphatidic acid phosphatase; DGAT —diacylglycerolacyl transferase.

Statistical analysis

Biplot was generated for eighteen genes as observations (Fig. 6). Based on the squared cosine values, it was revealed that the genes *ACP*, *KASII*, *KAR*, *HAD*, *ER*, *PT*, and *FATA* of FA

biosynthesis and *LPAAT, PAP*, and *DGAT* of TAG biosynthesis showed higher significance for high lipid content.

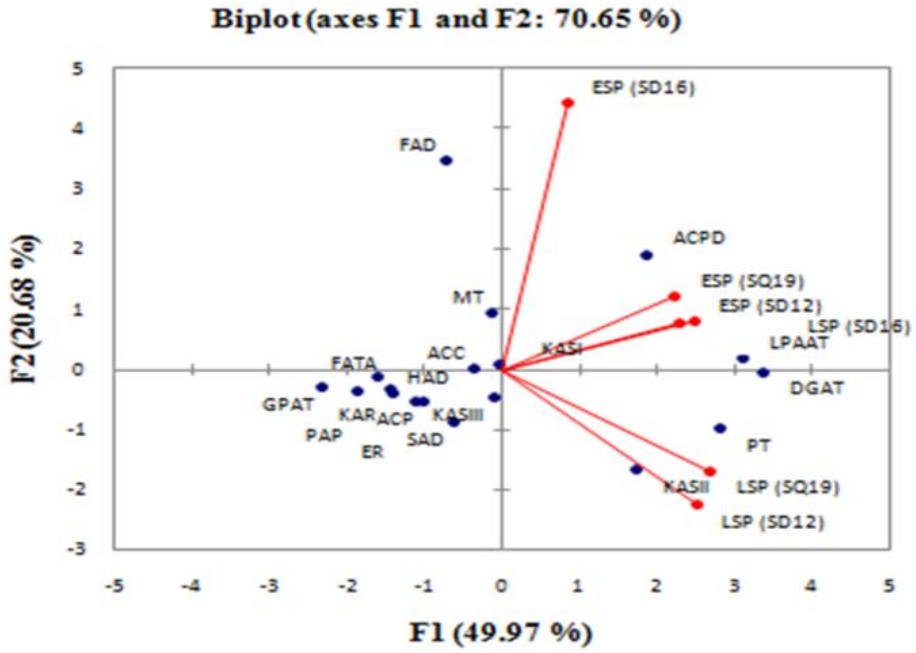

Fig. 6 Biplot for the respective PCA analysis of 18 lipid biosynthetic genes in growth stages among two strains of *S. dimoprhus* (SD16, SD12) and one *S. quadricauda* strain (SQ19)

A representative heat map demonstrating the differential expression of fatty acid and triacylgycerol biosynthetic pathway genes in various growth stages and stress conditions among three lipid contrasting *Scenedesmus* strains was generated using GenEx (Fig. 7).

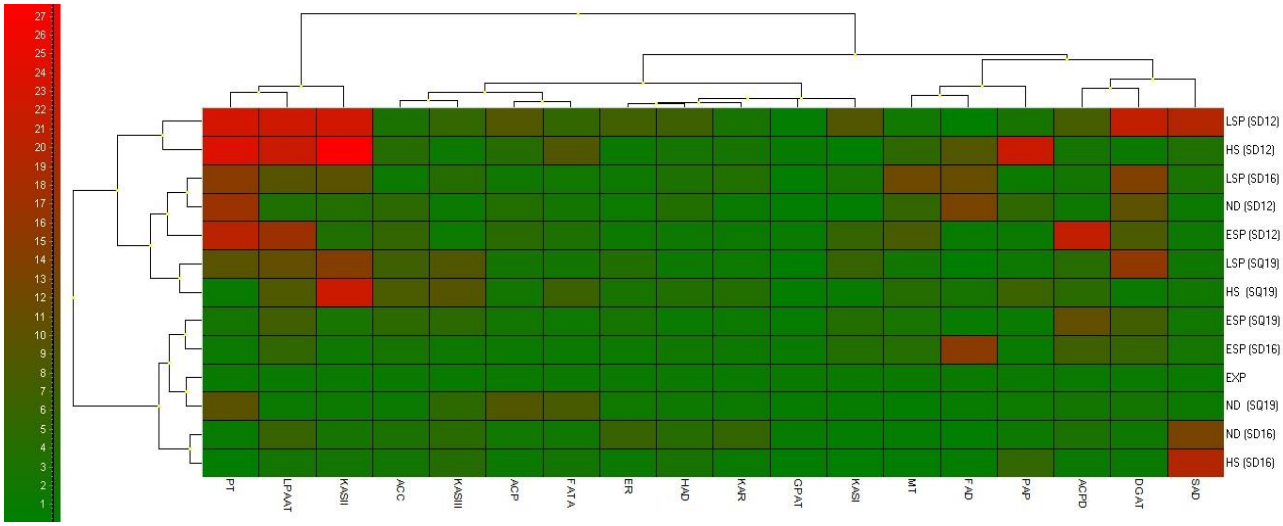

Fig. 7 A representative heatmap demonstrating the expression profiling of fatty acid and triacylglycerol biosynthetic pathway genes in various growth stages and stress conditions among three lipid contrasting *Scenedesmus* strains. Abbreviations: EXP—exponential phase, ESP—early stationary phase, LSP—late stationary phase,ND—nitrogen deficiency,HS—high salinity, ACC — acetyl-CoA carboxylase; MT — malonyl transferase; ACP — acyl carrier protein; KASI — β-ketoacyl-ACP synthase I; KASII — β-ketoacyl-ACP synthase II; KASIII — β-ketoacyl-ACP synthase III; KAR — β-ketoacyl-ACP reductase; HAD — β-hydroxyacyl ACP dehydratase; ER — enoyl-ACP eductase; PT — palmitoyl thioesterase; FATA — fatty acyl-ACP thioesterase; SAD — stearoyl-ACP desaturase; ACPD — palmitoyl desaturase; FAD — omega fatty acid desaturase; GPAT — glycerol-3-phosphate cyltransferase; LPAAT — lyso-phosphatidic acid acyltransferase; PAP — phosphatidic acid phosphatase; DGAT — diacylglycerolacyl transferase.

Conclusion

Out of 18 genes studied, expression of six genes of FA biosynthesis and three genes of TAG biosynthesis pathway was found to be positively correlated with the lipid content in all three strains suggesting their role in lipid accumulation. Further, the correlation between gene expression and lipid accumulating growth stages and conditions revealed key genes involved in storage lipid biosynthesis in *Scenedemus* sp. Genes *ACP, KASII, FATA, DGAT, PAP, LPAAT* can be considered as potential targets to design new strain improvement strategies by metabolic engineering in order to commercially utilize microalgae for biodiesel production.

Objective 2- Deciphering molecular components channeling precursors to differential lipid accumulation and their regulation in *Scenedesmus* species

Materials and methods

Library preparation for transcriptome analysis

Paired-end cDNA sequencing libraries were prepared using IlluminaTruSeq stranded total RNA library preparation kit according to the protocol described by the manufacturer. rRNA was depleted from total RNA followed by fragmentation. The fragmented rRNA depleted RNA was converted into first-strand cDNA, followed by second-strand cDNAsynthesis, A-tailing, adapter ligation and finally ended by index PCR amplification of adaptor-ligated library. Library quantification and validation was performed using QubitdsDNA HS kit and High Sensitivity Assay Kit, respectively.

De novo transcriptome assembly of S. dimoprhus and S. quadricauda

The raw data of two microalgal species were generated on NextSeq. The raw reads generated were filtered using Trimmomatic (v 0.30) with quality value QV > 20 and other contaminants. QC passed reads of the two species were subjected to *de novo* assembly with Trinity and final assembled transcripts were generated. CD-HIT-EST was run on the assembled transcripts to get unigenes. CDS were predicted from these unigenes using ORF Predictor (http://proteomics.ysu.edu/tools/OrfPredictor.html) with default parameters.

Functional annotation of predicted CDSs

Functional annotation of predicted CDSs from both the species was performed by aligning CDSs to non-redundant database of NCBI using BLASTx with E-value cut-off of 10^{-6} for both the species.

Pathway analysis

KAAS was used to functionally annotate the CDSs of *S. dimoprhus* and *S. quadricauda* by BLAST comparisons against KEGG GENES database. The BBH (bi-directional best hit) option was used to assign KO terms. The KEGG Orthology database (http://www.genome.jp/kegg/ko.html) was used for pathway mapping.

Transcript abundance estimation

Quantification of transcript abundance for the *de novo* assembled sequences was carried out using RSEM approach. Command rsem-prepare-reference was used to prepare the reference sequences and rsem-calculate-expression was used to calculate the transcript abundances.

Identification of transcription factors from transcriptomes of S. dimoprhus and S. quadricauda

Identified CDSs of *S. dimoprhus* and *S. quadricauda* were subjected to BLAST search against PlantTFDB (http://planttfdb.cbi.pku.edu.cn) with E-value cut-off of 10^{-5}. Only the top bit-scoring significant hit for each sequence was considered. Further, list of lipid related transcription factors from previous reports was used for the identification of lipid related transcription factors in *S. dimoprhus* and *S. quadricauda*.

Gene expression validation through RT-qPCR

RT-qPCR was performed using gene specific primers in triplicate on a RT-qPCRsystem (CFX96, Bio-Rad Laboratories; Hercules CA) with the iScript RT PCR kit (Bio-rad).

Results and discussion

Two species of *Scenedesmus* viz. *S. dimorphus* and *S. quadricauda*, having lipid contents of 26 % and 14%, respectively, were selected for transcriptomic analysis to dissect the molecular basis of cross species lipid content variation. Cultures of both the species were harvested at 20^{th} day for further analysis.

De novo sequence assembly

RNA-Seq libraries were prepared using Illumina sequencing. High quality reads were subjected to *de novo* assembly with Trinity resulting in 60,826 transcripts for *S. dimoprhus* and 145,112 transcripts for *S. quadricauda*. CD-HIT-EST was applied on the trinity assembled transcripts, which resulted in 42,979 unigenes in *S. dimoprhus* and 78,386 unigenes for *S. quadricauda*. 40,979 and 76,969 unigenes were successfully mapped into CDSs in *S. dimoprhus* and *S. quadricauda*, respectively.

Functional Annotation of Predicted CDSs

Total of 16,678 CDSs showed significant hits in *S. dimoprhus*, out of which 10,194 CDSs were annotated with green algal species. In case of *S. quadricauda*, 11,917 CDSs were considered as the best hits, out of which 11,917 CDSs were annotated with green algal species. It was found that the majority of significant hits were contributed from *Volvox carteri* followed by *Chlamydomonas reinhardtii* and *Chlorella variabilis* in both the species (Fig. 8).

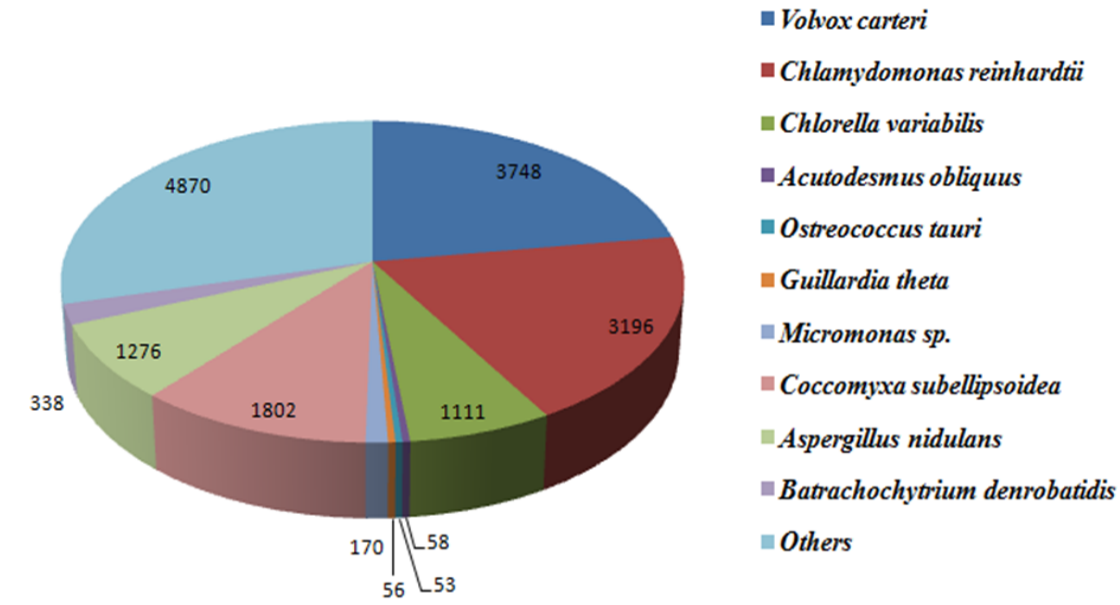

Fig. 8 A Top Blast species distribution for BLASTx matches in *S.dimorphus*

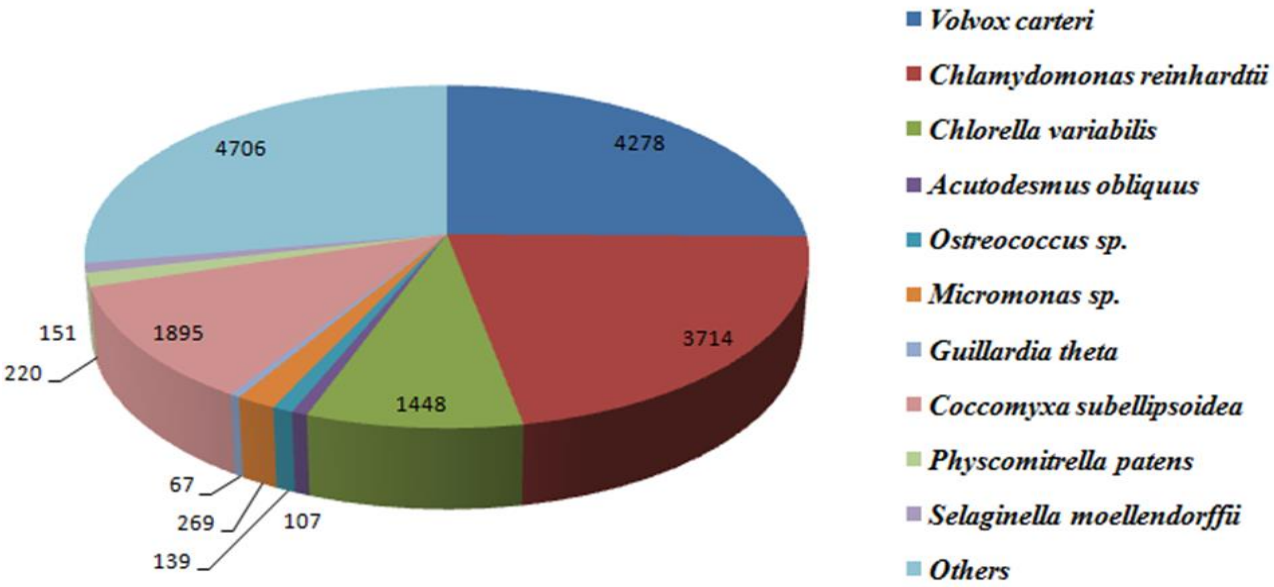

Fig. 8 B Top Blast species distribution for BLASTx matches in *S. quadricauda*.

Pathway classification by KEGG

KAAS was used for assigning KO identifiers to the predicted CDSs and subsequently mapping them to generate KEGG pathways. We have identified enzymes involved in major metabolic pathways, genetic information processing, environmental information processing, cellular processes and organism systems.

Differentially expressed genes in both the species

About 3,893 genes were expressed in the both species, whereas 1,530 genes were found exclusively in *S. dimoprhus* and 412 genes were present exclusively in *S. quadricauda*. Genes showing up-regulation and down-regulation among common genes were found to be involved in various metabolic pathways, including photosynthesis, carbon metabolism, fatty acid and triacylglycerol metabolism, and energy metabolism (Fig. 9).

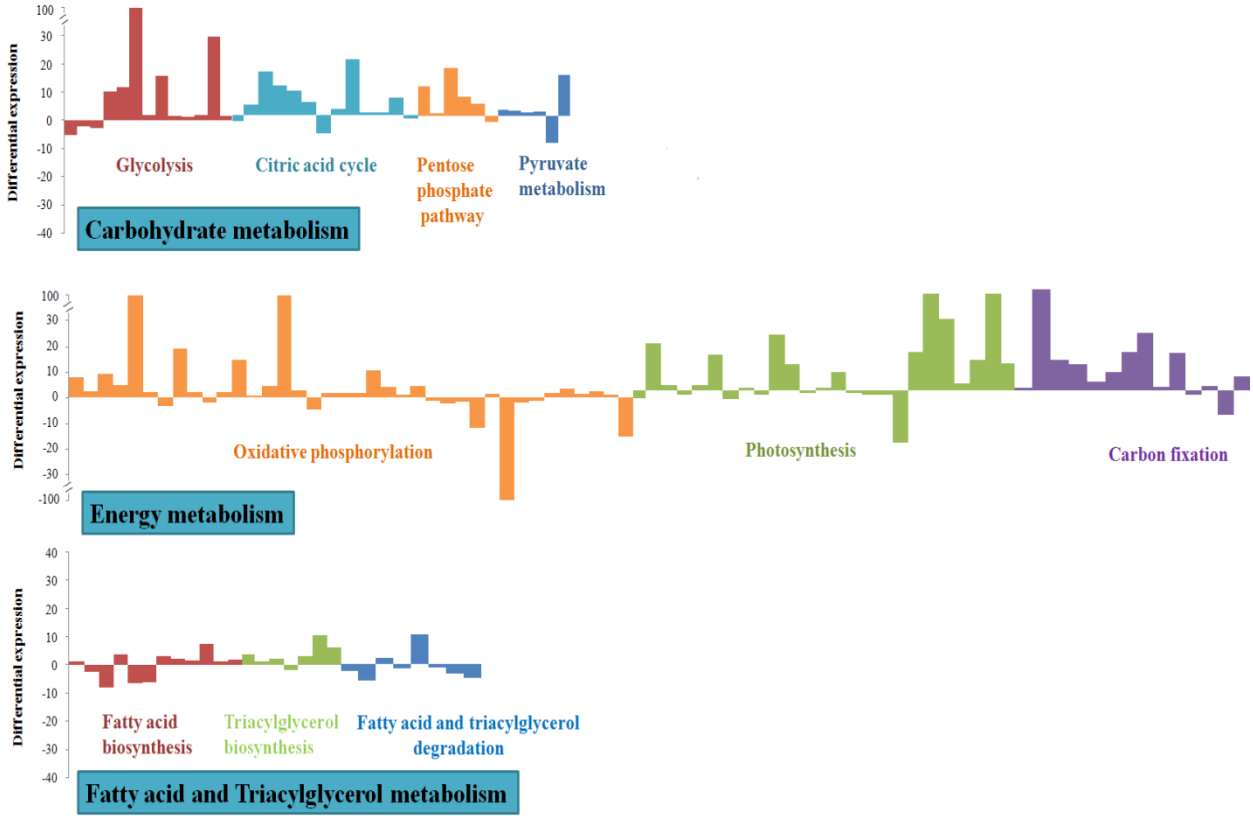

Fig. 9 Up-regulation and down-regulation of genes in various pathways of *S. dimoprhus* compared to *S. quadricauda*.

Enhanced photosynthesis in S. dimoprhus

About twenty six genes involved in photosynthesis were found common between both the species. Majority of the photosynthetic genes, including *PSI, PSII*, cytochrome b6 and light-harvesting complex proteins exhibited enhanced expression in *S. dimoprhus* compared to *S. quadricauda*. It has been reported earlier in *Chlorella vulgaris* that lipid accumulation improves with increase in photosynthetic light reactions [32].

Carbon metabolism provides precursors for storage lipid production

Almost all genes involved in carbon fixation showed enhanced expression in *S. dimoprhus* than in *S. quadricauda*. These included, ribulose-bisphosphate carboxylase large chain (*RBCL*), phosphoglycerate kinase (*PGK*), glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), fructose-bisphosphatealdolase (*ALDO*), fructose-1,6-bisphosphatase (*FBP*), sedoheptulose-bisphosphatase (*SBP*), transketolase (*TKT*), ribose 5-phosphate isomerase A (*RPIA*) and phosphoribulokinase (*PRK*). Increased expression of these genes in *S. dimoprhus* suggests the enhancement of carbon fixation process, thus excess carbon supply may have contributed in promoting the lipid biosynthesis and storage.

Further, gene encoding enzyme phosphoenolpyruvate carboxylase (*PEPC*) was found to be downregulated in *S. dimoprhus*. *PEPC* catalyzes the irreversible β-carboxylation of phosphoenolpyruvate (PEP) to produce inorganic phosphate and oxaloacetate [33]. Decreased

expression of *PEPC* resulted in an increase in the triacylglycerol content in *Chlamydomonas reinhartii* [34, 35] Therefore, down-regulation of *PEPC* in *S. dimoprhus* signifies its vital role for high lipid content of *S. dimoprhus*. Another gene of the glycolytic pathway, enolase (*ENO*) showed considerable increase of expression in *S. dimoprhus* (Fig. 8B). Increased expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and enolase (*ENO*) was reported in nitrogen deprived cultures of *Isochrysis galbana* leading to high lipid accumulation [36]. High expression of enolase was correlated with high oil accumulation in sunflower seeds, supported with its important role in carbon partitioning [37].

Another gene encoding enzyme citrate synthase (*CS*) was found to be down-regulated in *S. dimoprhus*. It has been reported that the suppression of citrate synthase resulted in increase of storage lipid content in *Chlamydomonas reinhardtii* [38]. Down-regulation of *CS* in *S. dimoprhus*, represents the shifting of carbon flux towards lipid production.

Acetyl-CoA synthetase (*ACOS*), pyruvate dehydrogenase (*PDH*) and ATP citrate lyase (*ACL*) are three enzymes involved in the production of acetyl-CoA, which is the main precursor of fatty acid synthesis, by alternative routes [39]. Genes encoding these three enzymes showed high transcript abundance in *S. dimoprhus*, suggesting their putative role in the regulation of storage lipid biosynthesis. Hence, overall up-regulation of genes providing precursors for fatty acid and triacylglycerol biosynthesis from different routes was observed in *S. dimoprhus*.

Comparative analysis of fatty acid and triacylglycerol biosynthetic pathways in S. dimoprhus and S. quadricauda

Majority of the genes, such as fatty acyl-ACP thioesterase A (*FATA*), oxoacyl-ACP synthase II (*KASII*), oxoacyl-ACP reductase (*KAR*), stearoyl-ACP desaturase (*SAD*) and hydroxyacyl-ACP dehydratase (*HAD*) showed up-regulation in *S. dimoprhus*. Glycerol kinase

(*GK*), the enzyme that catalyzes the formation of glycerol-3-phosphate from glycerol and provides substrate for triacylglycerol biosynthesis pathway [31] was found to be up-regulated in *S. dimoprhus*. Gene encoding glycerol-3-phosphate acyltransferase (*GPAT*) was down-regulated in *S. dimoprhus*. Other genes catalyzing the last steps of biosynthesis of triacylglycerol, i.e. phosphatidate phosphatase (*PAP*) and diacylglycerol acyltransferase (*DGAT*) were highly up-regulated in *S. dimoprhus*.

Further, gene encoding enzyme phospholipid: diacylglycerol acyltransferase (*PDAT*) has been identified in *S. dimoprhus* with a remarkable expression while it has not been found to be expressed in *S. quadricauda*, indicating another control point for high storage lipid accumulation in *S. dimoprhus*.

Table 1 Differentially expressed genes of carbon metabolism and fatty acid and triacylglycerol metabolism in *S. dimoprhus* and *S. quadricauda*

Gene symbol	**Gene name**	**EC number**	**Fold change**
Carbon metabolism			
HK	Hexokinase	2.7.1.1	- 0.25
GPI	Glucose-6-Phosphate isomerase	5.3.1.9	+ 0.62
PFK	Phosphofructokinase 1	2.7.1.11	- 0.50
ALDO	Fructose-bisphosphateAldolase	4.1.2.13	+ 13.65
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	1.2.1.12	+ 15.67
PGK	Phosphoglycerate kinase	2.7.2.3	+ 107.67
GPMI	Bisphosphoglycerate-independent phosphoglyceratemutase	5.4.2.12	+ 1.08
ENO	Enolase	4.2.1.11	+ 20.75
PK	Pyruvate kinase	2.7.1.40	- 1.88
FBP	Fructose-1,6-bisphosphatase	3.1.3.11	- 4.57
RBCL	Ribulose-bisphosphate carboxylase large chain	4.1.1.39	+ 1.48

TKT	Transketolase	2.2.1.1	+ 9.30
TAL	Transaldolase	2.2.1.2	+ 6.10
PPDK	Pyruvate, orthophosphate dikinase	2.7.9.1	+ 2.06
PEPC	Phosphoenolpyruvate carboxylase	4.1.1.31	- 0.14
PEPCK	Phosphoenolpyruvatecarboxykinase (ATP)	4.1.1.49	+ 7.04
MDH	Malate dehydrogenase	1.1.1.37	+ 8.27
ACOS	Acetyl-CoA synthetase	6.2.1.1	+ 1.86
CS	Citrate synthase	2.3.3.1	- 0.63
ACL	ATP citrate lyase	2.3.3.8	+ 1.01
PDH	Pyruvate dehydrogenase E1 component alpha subunit	1.2.4.1	+ 2.79
DLD	Dihydrolipoamide dehydrogenase	1.8.1.4	+ 1.53
G6PD	Glucose-6-phosphate 1-dehydrogenase	1.1.1.49	+ 41.96
PGD	Phosphogluconate dehydrogenase	1.1.1.343	+ 67.81
GAPN	Glyceraldehyde-3-phosphate dehydrogenase (NADP+)	1.2.1.9	+ 1.56
GAPA	Glyceraldehyde-3-phosphate dehydrogenase (NADP+) (phosphorylating)	1.2.1.13	+ 19.30
Fatty acid biosynthesis			
ACCA	Acetyl-CoA carboxylase carboxyl transferase subunit alpha	6.4.1.2	- 0.20
ACCC	Acetyl-CoA carboxylase, biotin carboxylase subunit	6.3.4.14	+ 2.77
ACCD	Acetyl-CoA carboxylase carboxyl transferase subunit beta	6.4.1.2	- 0.21
KAS III	3-oxoacyl-ACP synthase III	2.3.1.180	- 0.16
KAS II	3-oxoacyl-ACP synthase II	2.3.1.179	-1.14
KAR	3-oxoacyl-ACP reductase	1.1.1.100	+ 1.14
HAD	3-hydroxyacyl-ACP dehydratase	4.2.1.59	+ 4.00
FATA	Fatty acyl-ACP thioesterase	3.1.2.14	+ 5.45
EAR	Enoyl-ACP reductase	1.3.1.10	- 0.53
SAD	Stearoyl-ACP desaturase	1.14.19.2	+ 5.05
FAD	Omega-6 fatty acid desaturase	1.14.19.	- 0.64

Triacylglycerol biosynthesis			
GPAT	Glycerol-3-phosphate acyltransferase	2.3.1.15	- 0.35
PAP	Phosphatidate phosphatase	3.1.3.4	+ 41.94
DGAT	Diacylglycerolacyltransferase	2.3.1.20	+ 14.07
GK	Glycerol kinase	2.7.1.30	+1.70
Fatty acid and triacylglycerol degradation			
ACSL	Long-chain acyl-CoA synthetase	6.2.1.3	- 0.47
MFP	Enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase	4.2.1.17	- 0.61
ACOX	Acyl-CoA oxidase	1.3.3.6	- 0.50
ACD	Acyl-CoA dehydrogenase	1.3.8.7	- 0.22
ACAT	Acetyl-CoA acetyltransferase	2.3.1.9	+ 2.93
TGL	TAG lipase	3.1.1.3	- 0.27

(+: Up-regulation; - : Down-regulation)

Transcription factor WRI1 correlates positively with high lipid production in S. dimoprhus

Total of 613 and 454 CDSs encoding transcription factors belonging to 25 TF families were found in *S. dimoprhus* and *S. quadricauda*, respectively. The most abundant TFs in both the species were found to be of MYB TF family, including MYB and MYB-related families. These transcription factors are involved in the regulation of gene expression, secondary metabolism and responses to environmental stresses [40]. Also, over expression of soybean MYB in transgenic *Arabidopsis* plants resulted in increased lipid content [41].

Further, literature based mining was performed to identify CDSs encoding transcription factors associated with lipid metabolism in both the species. Hu *et al.* identified thirty transcription factors involved in lipid metabolism by *in-silico* approach that belong to various TF families, viz. AP2, HB-other, C3H, MYB_related, E2F/DP, MYB, NF-YC, CPP, HSF, bZIP, LFY [22]. Out of these, twenty transcription factors were identified in *S. dimoprhus*, whereas only eight were

identified in *S. quadricauda*. Seven transcription factors were common, out of which five were found to be up-regulated belonging to TF families i.e. AP2, MYB, LFY and NF-YC. It was observed that one up-regulated transcription factor of AP2 family has shown considerable fold increase of expression in *S. dimoprhus* than in *S. quadricauda*. This transcription factor was found to be WRINKLED1 (WRI1) ortholog, which governs fatty acid synthesis and triacylglycerol accumulation in various plant species [42-44].

Experimental validation of in-silico transcript abundance by RT-qPCR

Seven key genes identified to be involved in supplying precursors to fatty acid and triacylglycerol biosynthesis were further validated in both high lipid content and low lipid content species of *Scenedesmus* using RT-qPCR. All genes showed positive correlation with *in-silico* transcript abundance analysis. Further, the expression of CDS encoding transcription factor WRI1 was analysed by RT-qPCR in both the species at three growth stages. There is a considerable fold difference in the expression of WRI1 in *S. dimoprhus* and *S. quadricauda* as confirmed by RT-qPCR. In three growth stages, expression of WRI1 showed a dramatic increase at the start of stationary phase in *S. dimoprhus* whereas in *S. quadricauda*, slight increase in the expression was observed at stationary phase. RT-qPCR results confirmed the reliability of computational analysis of RNA-Seq data and identified genes governing differential lipid accumulation in both the species (Fig. 10).

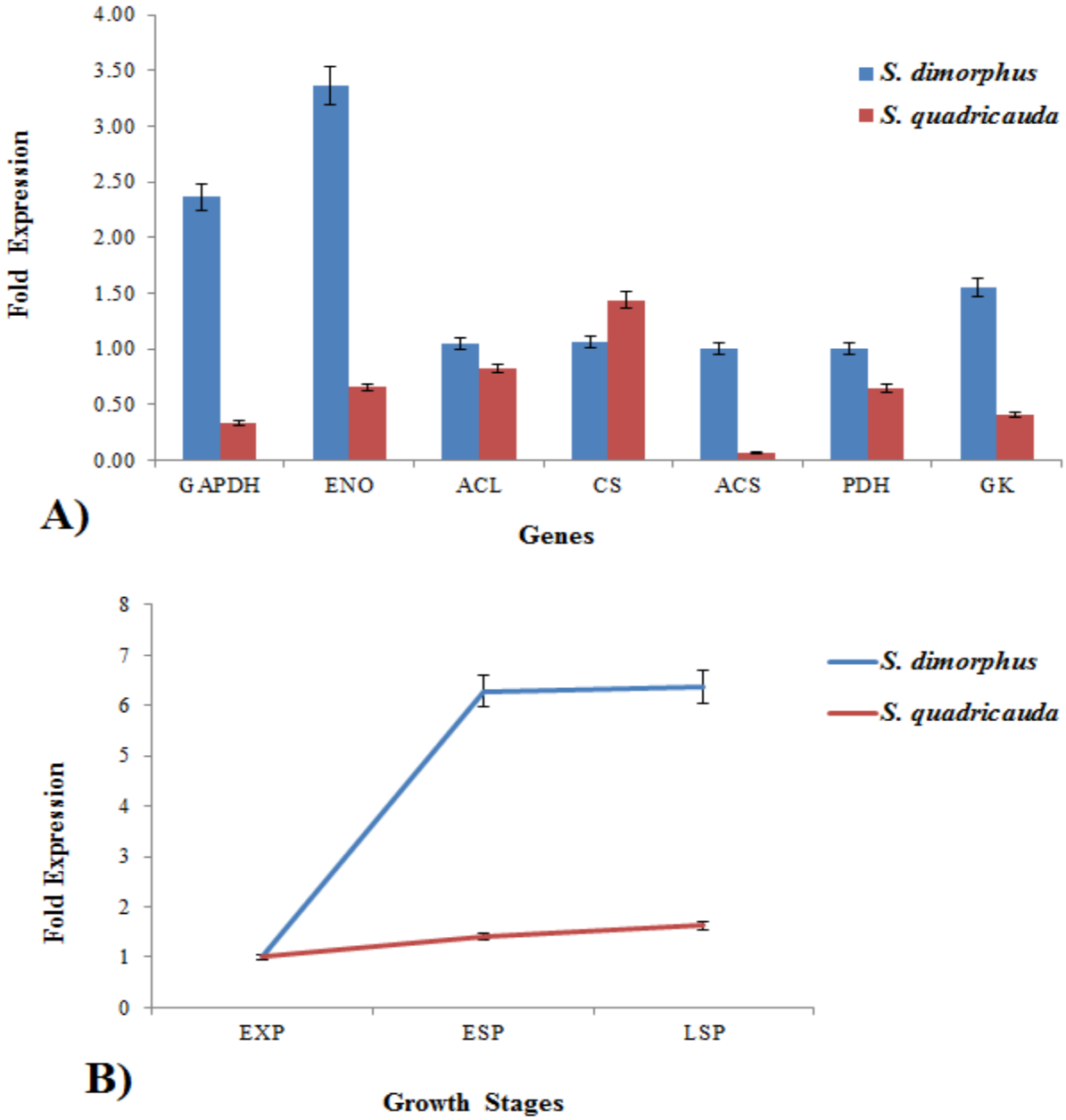

Fig. 10 A) Expression analysis of seven identified key genes in *S. dimoprhus* and *S. quadricauda* at stationary phase by RT-qPCR. Abbreviations: GAPDH -Glyceraldehyde 3-phosphate dehydrogenase; ENO- Enolase; ACOS- Acetyl-CoA synthetase; PDH - Pyruvate dehydrogenase; ACL- ATP citrate lyase; GK- Glycerol kinase; CS- Citrate synthase **B)** Relative expression analysis of CDS encoding transcription factor WRINKLED1in *S. dimoprhus* and *S. quadricauda* in three growth stages.

Conclusion

High lipid accumulation was found to be correlated with certain pathways including, photosynthesis, carbon fixation, glycolysis, pyruvate metabolism, TCA cycle, fatty acid and triacylglycerol biosynthesis. Key genes of carbon metabolism, glyceraldehyde 3-phosphate dehydrogenase, enolase, acetyl-CoA synthetase, pyruvate dehydrogenase, ATP citrate lyase, glycerol kinase, citrate synthase were identified as major control points for high lipid content in *S. dimorphus.* These genes provide carbon precursors to triacylglycerol biosynthesis and therefore, can be targeted by metabolic engineering approaches for enhanced lipid production. Correlation of WRI1 transcription factor with high lipid accumulation was inferred from the comparative analysis between *S. dimoprhus* and *S. quadricauda*.

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Publications

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